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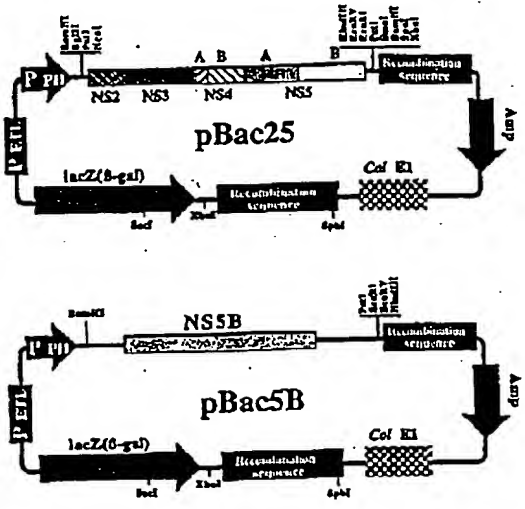
(54) Title: METHOD FOR REPRODUCING IN VITRO THE RNA-DEPENDENT RNA POLYMERASE AND TERMINAL NUCLEOTIDYL TRANSFERASE ACTIVITIES ENCODED BY HEPATITIS C VIRUS (HCV)

(57) Abstract

This is a method for reproducing in vitro the RNA-dependent RNA polymerase activity associated with hepatitis C virus. The method is characterized in that sequences contained in NS5B are used in the reaction mixture. The terminal nucleotidyl transferase activity, a further property of the NS5B protein, can also be reproduced using this method. The method takes advantage of the fact that the NS5B protein, either purified to apparent homogeneity or present in extracts of overproducing organisms, can catalyse the addition of ribonucleotides to the 3'-termini of exogenous or endogenous RNA molecules. The invention also relates to a composition of matter that comprises sequences contained in NS5B, and to the use of these compositions for the set up of an enzymatic test capable of selecting, for therapeutic purposes, compounds that inhibit the enzymatic activity associated with NS5B. The figure shows plasmids used in the method to produce hepatitis C virus RNA-dependent RNA polymerase and terminal nucleotidyl transferase in cultivated eukaryotic and prokaryotic cells.



P ETL = promoter of the gene coding for the PCNA protein

P PH = promoter of the polyhedrin gene

Amp = gene coding for the ß-lactamase enzyme (ampicillin resistance)

LacZ (ß-gal) = gene coding for the ß-galactosidase enzyme

Col E1 = pBR322 replication origin

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METHOD FOR REPRODUCING IN VITRO THE RNA-DEPENDENT RNA POLYMERASE AND TERMINAL NUCLEOTIDYL TRANSFERASE ACTIVITIES ENCODED BY HEPATITIS C VIRUS (HCV)

DESCRIPTION

The present invention relates to the molecular biology and virology of the hepatitis C virus (HCV). More specifically, this invention has as its object the RNA-dependent RNA polymerase (RdRp) and the nucleotidyl terminal transferase (TNTase) activities produced by HCV, methods of expression of the HCV RdRp and TNTase, methods for assaying *in vitro* the RdRp and TNTase activities encoded by HCV in order to identify, for therapeutic purposes, compounds that inhibit these enzymatic activities and therefore might interfere with the replication of the HCV virus.

As is known, the hepatitis C virus (HCV) is the main etiological agent of non-A, non-B hepatitis (NANB). It is estimated that HCV causes at least 90% of post-transfusional NANB viral hepatitis and 50% of sporadic NANB hepatitis. Although great progress has been made in the selection of blood donors and in the immunological characterization of blood used for transfusions, there is still a high number of HCV infections among those receiving blood transfusions (one million or more infections every year throughout the world). Approximately 50% of HCV-infected individuals develop cirrhosis of the liver within a period that can range from 5 to 40 years. Furthermore, recent clinical studies suggest that there is a correlation between chronic HCV infection and the development of hepatocellular carcinoma.

HCV is an enveloped virus containing an RNA positive genome of approximately 9.4 kb. This virus is a member of the Flaviviridae family, the other embers of which are the flaviviruses and the pestiviruses. The RNA genome of HCV has recently been mapped. Comparison of sequences from the HCV genomes isolated in various parts of the

world has shown that these sequences can be extremely heterogeneous. The majority of the HCV genome is occupied by an open reading frame (ORF) that can vary between 9030 and 9099 nucleotides. This ORF codes for a single viral polyprotein, the length of which can vary from 3010 to 3033 amino acids. During the viral infection cycle, the polyprotein is proteolytically processed into the individual gene products necessary for replication of the virus. The genes coding for HCV structural proteins are located at the 5'-end of the ORF, whereas the region coding for the non-structural proteins occupies the rest of the ORF.

The structural proteins consist of C (core, 21 kDa), E1 (envelope, gp37) and E2 (NS1, gp61). C is a non-glycosylated protein of 21 kDa which probably forms the viral nucleocapsid. The protein E1 is a glycoprotein of approximately 37 kDa, which is believed to be a structural protein for the outer viral envelope. E2, another membrane glycoprotein of 61 kDa, is probably a second structural protein in the outer envelope of the virus.

The non-structural region starts with NS2 (p24), a hydrophobic protein of 24 kDa whose function is unknown. NS3, a protein of 68 kDa which follows NS2 in the polyprotein, is predicted to have two functional domains: a serine protease domain in the first 200 amino-terminal amino acids, and an RNA-dependent ATPase domain at the carboxy terminus. The gene region corresponding to NS4 codes for NS4A (p6) and NS4B (p26), two hydrophobic proteins of 6 and 26 kDa, respectively, whose functions have not yet been clarified. The gene corresponding to NS5 also codes for two proteins, NS5A (p56) and NS5B (p65), of 56 and 65 kDa, respectively.

Various molecular biological studies indicate that the signal peptidase, a protease associated with the endoplasmic reticulum of the host cell, is responsible for proteolytic processing in the non-structural region,

that is to say at sites C/E1, E1/E2 and E2/NS2. A virally-encoded protease activity of HCV appears to be responsible for the cleavage between NS2 and NS3. This protease activity is contained in a region comprising both part of NS2 and the part of NS3 containing the serine protease domain, but does not use the same catalytic mechanism. The serine protease contained in NS3 is responsible for cleavage at the junctions between S3 and NS4A, between NS4A and NS4B, between NS4B and NS5A and between NS5A and NS5B.

Similarly to other (+)-strand RNA viruses, the replication of HCV is thought to proceed *via* the initial synthesis of a complementary (-)-RNA strand, which serves, in turn, as template for the production of progeny (+)-strand RNA molecules. An RNA-dependent RNA polymerase (RdRp) has been postulated to be involved in both these steps. An amino acid sequence present in all the RNA-dependent RNA polymerases can be recognized within the NS5 region. This suggests that the NS5 region contains components of the viral replication machinery. Virally-encoded polymerases have traditionally been considered important targets for inhibition by antiviral compounds. In the specific case of HCV, the search for such substances has, however, been severely hindered by the lack of both a suitable model system of viral infection (e.g. infection of cells in culture or a facile animal model), and a functional RdRp enzymatic assay.

It has now been unexpectedly found that this important limitation can be overcome by adopting the method according to the present invention, which also gives additional advantages that will be evident from the following.

The present invention has as its object a method for reproducing in vitro the RNA-dependent RNA polymerase activity of HCV that makes use of sequences contained in the HCV NS5B protein. The terminal nucleotidyl transferase activity, a further property of the NS5B

protein, can also be reproduced using this method. The method takes advantage of the fact that the proteins containing sequences of NS5B can be expressed in either eukaryotic or prokaryotic heterologous systems: the recombinant proteins containing sequences of NS5B, either purified to apparent homogeneity or present in extracts of overproducing organisms, can catalyse the addition of ribonucleotides to the 3'-termini of exogenous RNA molecules, either in a template-dependent (RdRp) or template-independent (TNTase) fashion.

The invention also extends to a new composition of matter, characterized in that it comprises proteins whose sequences are described in SEQ ID NO: 1 or sequences contained therein or derived therefrom. It is understood that this sequence may vary in different HCV isolates, as all the RNA viruses show a high degree of variability. This new composition of matter has the RdRp activity necessary to the HCV virus in order to replicate its genome.

The present invention also has as its object the use of this composition of matter in order to prepare an enzymatic assay capable of identifying, for therapeutic purposes, compounds that inhibit the enzymatic activities associated with NS5B, including inhibitors of the RdRp and that of the TNTase.

Up to this point a general description has been given of the present invention. With the aid of the following examples, a more detailed description of specific embodiments thereof will now be given, in order to give a clearer understanding of its objects, characteristics, advantages and method of operation.

Figure 1 shows the plasmids constructs used for the transfer of HCV cDNA into a baculovirus expression vector.

Figure 2 shows the plasmids used for the in vitro synthesis of the D-RNA substrate of the HCV RNA-dependent RNA polymerase [pT7-7(DCoH)], and for the expression of

the HCV RNA-dependent RNA polymerase in E. coli cells [pT7-7(NS5B)], respectively.

Figure 3 shows a schematic drawing of (+) and (-) strands of D-RNA. The transcript contains the coding region of the DCoH mRNA. The DNA-oligonucleotides a, b and c were designed to anneal with the newly-synthesized antisense RNA and the DNA/RNA hybrid was subjected to cleavage with RNase H. The lower part of the scheme depicts the expected RNA fragment sizes generated by RNase digestion of the RNA (-) hybrid with oligonucleotides a, b and c, respectively.

DEPOSITS

E. Coli DH1 bacteria, transformed using the plasmids pBac 5B, pBac 25, pT7.7 DCoH and pT7.7NS5B - containing SEQ ID NO:1; SEQ ID NO:2; the cDNA for transcription of SEQ ID NO:12; and SEQ ID NO:1, respectively, filed on May 9, 1995 with The National Collections of Industrial and Marine Bacteria Ltd. (NCIMB), Aberdeen, Scotland, UK. under access numbers NCIMB 40727, 40728, 40729 and 40730, respectively.

EXAMPLE 1

Method of expression of HCV RdRp/TNTase in *Spodoptera frugiperda* clone 9 (Sf9) cultured cells.

Systems for expression of foreign genes in insect cultured cells, such as *Spodoptera frugiperda* clone 9 (Sf9) cells infected with baculovirus vectors are known in the art (V. A. Luckow, Baculovirus systems for the expression of human gene products, (1993) Current Opinion in Biotechnology 4, pp. 564-572). Heterologous genes are usually placed under the control of the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus of the *Bombix mori* nuclear polyhedrosis virus. Methods for the introduction of heterologous DNA in the desired site in the baculoviral vectors by homologous recombination are also known in the art (D.R. O'Reilly, L. K. Miller, V.A. Luckow, (1992),

Baculovirus Expression Vectors-A Laboratory Manual, W. H. Freeman and Company, New York).

Plasmid vectors pBac5B and pBac25 are derivatives of a derivative of pBlueBacIII (Invitrogen) and were constructed for transfer of genes coding for NS4B and other non-structural HCV proteins in baculovirus expression vectors. The plasmids are schematically illustrated in figure 1 and their construction is described in detail in Example 8. Selected fragments of the cDNA corresponding to the genome of the HCV-BK isolate (HCV-BK; Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I. and Okayama, H., (1991) Structure and Organization of the Hepatitis C Virus Genome Isolated from Human Carriers *J. Virol.*, 65, 1105-1113) were cloned under the strong polyhedrin promoter of the nuclear polyhedrosis virus and flanked by sequences that allowed homologous recombination in a baculovirus vector.

In order to construct pBac5B, a PCR product containing the cDNA region encoding amino acids 2420 to 3010 of the HCV polyprotein and corresponding to the NS5B protein (SEQ ID NO:1) was cloned between the *Bam*HI and *Hin*dIII sites of pBlue BacIII. The PCR sense oligonucleotide contained a translation initiation signal, whereas the original HCV termination codon serves for translation termination.

pBac25 is a derivative of pBlueBacIII (Invitrogen) where the cDNA region coding for amino acids 810 to 3010 of the HCV-BK polyprotein (SEQ ID NO:2) was cloned between the *Nco*I and the *Hin*dIII restriction sites.

Spodoptera frugiperda clone 9 (Sf9) cells and baculovirus recombination kits were purchased from Invitrogen. Cells were grown on dishes or in suspension at 27°C in complete Grace's insect medium (Gibco) containing 10% foetal bovine serum (Gibco). Transfection, recombination, and selection of baculovirus constructs were performed as recommended by the

manufacturer. Two recombinant baculovirus clones, Bac25 and Bac5B, were isolated that contained the desired HCV cDNA.

For protein expression, Sf9 cells were infected either with the recombinant baculovirus Bac25 or Bac5B at a density of 2 x 10^6 cells per ml in a ratio of about 5 virus particles per cell. 48-72 hours after infection, the Sf9 cells were pelleted, washed once with phosphate buffered saline (PBS) and carefully resuspended (7.5 x 10^7 cells per ml) in buffer A (10 mM Tris/Cl pH 8, 1.5 mM $MgCl_2$, 10 mM NaCl) containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl-fluoride (PMSF, Sigma) and 4 mg/ml leupeptin. All the following steps were performed on ice: after swelling for 30 minutes, the cells were disrupted by 20 strokes in a Dounce homogeniser using a tight-fitting pestle. Glycerol, as well as the detergents Nonidet P-40 (NP40) and 3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), were added to final concentrations of 10% (v/v), 1% (v/v) and 0.5% /w/v), respectively, and the cellular extract was incubated for a further hour on ice with occasional agitation. The nuclei were pelleted by centrifugation for 10 minutes at 1000 x g, and the supernatant was collected. The pellet was resuspended in buffer A containing the above concentrations of glycerol and detergents (0.5 ml per 7.5 x 10^7 nuclei) by 20 strokes in the Dounce homogeniser and then incubated for one hour on ice. After repelleting the nuclei, both supernatants were combined, centrifuged for 10 minutes at 8000 x g and the pellet was discarded. The resulting crude cytoplasmic extract was used either directly to determine the RdRp activity or further purified on a sucrose gradient (see Example 5).

Infection of Sf9 cells with either the recombinant baculovirus Bac25 or Bac5B leads to the expression of the expected HCV proteins. Indeed, following infection of Sf9 cells with Bac25, correctly-processed HCV NS2 (24

kDa), NS3 (68 kDa), NS4B (26 kDa), NS4A (6 kDa), NS5A (56 kDa) and NS5B (65 kDa) proteins can be detected in the c 11 lysates by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunostaining. Following infection of Sf9 cells with Bac5B, only one HCV-encoded protein, corresponding in size to authentic NS5B (65 kDa), is detected by SDS-PAGE followed by immuno- or Coomassie Blue staining.

EXAMPLE 2

Method of assay of recombinant HCV RdRp on a synthetic RNA template/substrate.

The RdRp assay is based on the detection of labelled nucleotides incorporated into novel RNA products. The *in vitro* assay to determine RdRp activity was performed in a total volume of 40 µl containing 1-5 µl of either Sf9 crude cytoplasmic extract or purified protein fraction. Unfractionated or purified cytoplasmic extracts of Sf9 cells infected with Bac25 or Bac5B may be used as the source of HCV RdRp. A Sf9 cell extract obtained from cells infected with a recombinant baculovirus construct expressing a protein that is not related to HCV may be used as a negative control. The following supplements are added to the reaction mixture (final concentrations): 20 mM Tris/Cl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 25 mM KCl, 1 mM EDTA, 5-10 µCi [^{32}P] NTP of one species (unless otherwise specified, GTP, 3000 Ci/mmol, Amersham, was used), 0.5 mM each NTP (i.e. CTP, UTP, ATP unless specified otherwise), 20 U RNasin (Promega), 0.5 µg RNA-substrate (ca. 4 pmol; final concentration 100 nM), 2 µg actinomycin D (Sigma). The reaction was incubated for two hours at room temperature, stopped by the addition of an equal volume of 2 x Proteinase K (PK, Boehringer Mannheim) buffer (300 mM NaCl, 100 mM Tris/Cl pH 7.5, 1% w/v SDS) and followed by half an hour of treatment with 50 µg of PK at 37°C. RNA products were PCA extracted, precipitated with ethanol and analysed by electrophoresis on 5% polyacrylamide gels containing 7M urea.

The RNA substrate we normally used for the assay (D-RNA) had the sequence reported in SEQ ID NO: 12, and was typically obtained by *in vitro* transcription of the linearized plasmid pT7-7(DCoH) with T7 polymerase, as described below.

Plasmid pT7-7(DCoH) (figure 2) was linearized with the unique *Bgl*II restriction site contained at the end of the DCoH coding sequence and transcribed *in vitro* with T7 polymerase (Stratagene) using the procedure described by the manufacturer. Transcription was stopped by the addition of 5 U/10µl of DNaseI (Promega). The mixture was incubated for a further 15 minutes and extracted with phenol/chloroform/ isoamylalcohol (PCA). Unincorporated nucleotides were removed by gel-filtration through a 1-ml Sephadex G50 spun column. After extraction with PCA and ethanol precipitation, the RNA was dried, redissolved in water and its concentration determined by optical density at 260 nm.

As will be clear from the experiments described below, any other RNA molecule other than D-RNA, may be used for the RdRp assay of the invention.

The above described HCV RdRp assay gave rise to a characteristic pattern of radioactively-labelled reaction products: one labelled product, which comigrated with the substrate RNA was observed in all reactions, including the negative control. This RNA species could also be visualised by silver staining and was thus thought to correspond to the input substrate RNA, labelled most likely by terminal nucleotidyl transferase activities present in cytoplasmic extracts of baculovirus-infected Sf9 cells. In the reactions carried out with the cytoplasmic extracts of Sf9 cells infected with either Bac25 or Bac5B, but not of cells infected with a recombinant baculovirus construct expressing a protein that is not related to HCV, an additional band was observed, migrating faster than the substrate RNA. This latter reaction product was found to be labelled to a

high specific activity, since it could be detected solely by autoradiography and not by silver staining. This novel product was found to be derived from the externally-added RNA template, as it was absent from control reactions where no RNA was added. Interestingly, the formation of a labelled species migrating faster than the substrate RNA was consistently observed with a variety of template RNA molecules, whether containing the HCV 3'-untranslated region or not. The 399 nucleotide mRNA of the liver-specific transcription cofactor DCoH (D-RNA) turned out to be an efficiently accepted substrate in our RdRp assay.

In order to define the nature of the novel species generated in the reaction by the Bac25- or Bac5B-infected cell extracts, we carried out the following series of experiments. (i) The product mixture was treated with RNAse A or Nuclease P1. As this resulted in the complete disappearance of the radioactive bands, we concluded that both the labelled products were RNA molecules. (ii) Omission from the reaction mixtures of any of the four nucleotide triphosphates resulted in labelling of only the input RNA, suggesting that the faster migrating species is a product of a polymerisation reaction. (iii) Omission of Mg^{2+} ions from the assay caused a complete block of the reaction: neither synthesis of the novel RNA nor labelling of the input RNA were observed. (iv) When the assay was carried out with a radioactively labelled input RNA and unlabelled nucleotides, the labelled product was indistinguishable from that obtained under the standard conditions. We concluded from this result that the novel RNA product is generated from the original input RNA molecule.

Taken together, our data demonstrate that the extracts of Bac25- or Bac5B-infected Sf9 cells contain a novel magnesium-dependent enzymatic activity that catalyses *de novo* RNA synthesis. This activity was shown to be dependent on the presence of added RNA, but

independent of an added primer or of the origin of the input RNA molecule. Moreover, as the products generated by extracts of Sf9 cells infected with either Bac25 or Bac5B appeared to be identical, the experiments just described indicate that the observed RdRp activity is encoded by the HCV NS5B protein.

EXAMPLE 3

Methods for the characterization of the HCV RdRp RNA product

The following methods were employed in order to elucidate the structural features of the newly-synthesized RNA product. Under our standard electrophoresis conditions (5% polyacrylamide, 7M urea), the size of the novel RNA product appeared to be approximately 200 nucleotides. This could be due to either internal initiation of RNA transcription, or to premature termination. These possibilities, however, appeared to be very unlikely, since products derived from RdRp assays using different RNA substrates were all found to migrate significantly faster than their respective templates. Increasing the temperature during electrophoresis and the concentration of acrylamide in the analytical gel lead to a significantly different migration behaviour of the RdRp product. Thus, using for instance a gel system containing 10% acrylamide, 7M urea, where separation was carried out at higher temperature, the RdRp product migrated slower than the input substrate RNA, at a position corresponding to at least double the length of the input RNA. A similar effect was observed when RNA-denaturing agents such as methylhydroxy-mercury (CH_3HgOH, 10 mM) were added to the RdRp products prior to electrophoresis on a low-percentage/lower temperature gel. These observations suggest that the RdRp product possesses an extensive secondary structure.

We investigated the susceptibility of the product molecule to a variety of ribonucleases of different specificity. The product was completely degraded upon

treatment with RNase A. On the other hand, it was found to be surprisingly resistant to single-strand specific nuclease RNase T1. The input RNA was completely degraded after 10 minutes incubation with 60 U RNase T1 at 22°C and silver staining of the same gel confirmed that not only the template, but also all other RNA usually detectable in the cytoplasmic extracts of Sf9 cells was completely hydrolysed during incubation with RNAse T1. In contrast, the RdRp product remained unaltered and was affected only following prolonged incubation with RNase T1. Thus, after two hours of treatment with RNase T1, the labelled product molecule could no longer be detected at its original position in the gel. Instead, a new band appeared that had an electrophoretic mobility similar to the input template RNA. A similar effect was observed when carrying out the RNAse T1 digestion for 1 hour, but at different temperatures: at 22°C, the RdRp product remained largely unaffected whereas at 37°C it was converted to the new product that co-migrates with the original substrate.

The explanation for these observations is that the input RNA serves as a template for the HCV RdRp, where the 3'-OH is used to prime the synthesis of the complementary strand by a turn-or "copy-back" mechanism to give rise to a duplex RNA "hairpin" molecule, consisting of the sense (template) strand to which an antisense strand is covalently attached. Such a structure would explain the unusual electrophoretic mobility of the RdRp product on polyacrylamide gels as well as its high resistance to single-strand specific nucleases. The turn-around loop should not be base-paired and therefore ought to be accessible to the nucleases. Treatment with RNase T1 thus leads to the hydrolysis of the covalent link between the sense and antisense strands to yield a double-stranded RNA molecule. During denaturing gel electrophoresis the two strands become separated and only the newly-synthesized

antisense strand, which should be similar in length to the original RNA template, would remain detectable. This mechanism would appear rather likely, especially in view of the fact that this kind of product is generated by several other RNA polymerases *in vitro*.

The following experiment was designed in order to demonstrate that the RNA product labelled during the polymerase reaction and apparently released by RNase T1 treatment exhibits antisense orientation with respect to the input template. For this purpose, we synthesized oligodeoxyribonucleotides corresponding to three separate sequences of the input template RNA molecule (figure 2), oligonucleotide a, corresponding to nucleotides 170-195 of D-RNA (SEQ ID NO: 3); oligonucleotide b, complementary to nucleotides 286-309 (SEQ ID NO: 4); oligonucleotide c, complementary to nucleotides 331-354 (SEQ ID NO: 5). These were used to generate DNA/RNA hybrids with the product of the polymerase reaction, such that they could be subjected to RNase H digests. Initially, the complete RdRp product was used in the hybridizations. However, as this structure is too thermostable, no specific hybrids were formed. The hairpin RNA was therefore pre-treated with RNase T1, denatured by boiling for 5 minutes and then allowed to cool down to room temperature in the presence of the respective oligonucleotide. As expected, exposure of the hybrids to RNase H yielded specific cleavage products. Oligonucleotide a-directed cleavage lead to products of about 170 and 220 nucleotides in length, oligonucleotide b yielded products of about 290 and 110 nucleotides and oligonucleotide c gave rise to fragments of about 330 and 65 nucleotides. As these fragments have the expected sizes (see figure 3), the results indicate that the HCV NS5B-mediated RNA synthesis proceeds by a copy-back mechanism that generates a hairpin-like RNA duplex.

EXAMPLE 4

Method of assay of recombinant HCV TNTase on a synthetic RNA substrate

The TNTase assay is based on the detection of template-independent incorporation of labelled nucleotides to the 3' hydroxyl group of RNA substrates. The RNA substrate for the assay (D-RNA) was typically obtained by *in vitro* transcription of the linearized plasmid pT7-7DCOH with T7 polymerase as described in Example 2. However, any other RNA molecule, other than D-RNA, may be used for the TNTase assay of the invention.

The in vitro assay to determine TNTase activity was performed in a total volume of 40 µl containing 1-5 µl of either Sf9 crude cytoplasmic extract or purified protein fraction. Unfractionated or purified cytoplasmic extracts of Sf9 cells infected with Bac25 or Bac5B may be used as the source of HCV TNTase. An Sf9 cell extract obtained from cells infected with a recombinant baculovirus construct expressing a protein that is not related to HCV may be used as a negative control. The following supplements are added to the reaction mixture (final concentrations): 20 mM Tris/Cl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 25 mM KCl, 1 mM EDTA, 5-10 µCi [^{32}P] NTP of one species (unless otherwise specified, UTP, 3000 Ci/mmol, Amersham, was used), 20 U RNasin (Promega), 0.5 µg RNA-substrate (ca. 4 pmol; final concentration 100 nM), 2 µg actinomycin D (Sigma). The reaction was incubated for two hours at room temperature, stopped by the addition of an equal volume of 2 x Proteinase K (PK, Boehringer Mannheim) buffer (300 mM NaCl, 100 mM Tris/Cl pH 7.5, 1% w/v SDS) and followed by half an hour of treatment with 50 µg of PK at 37°C. RNA products were PCA extracted, precipitated with ethanol and analysed by electrophoresis on 5% polyacrylamide gels containing 7M urea.

EXAMPLE 5

Method for the purification of the HCV RdRp/TNTase by sucrose gradient sedimentation

A linear 0.3-1.5 M sucrose gradient was prepared in buffer A containing detergents (see Example 1). Up to 2 ml of extract of Sf9 cells infected with Bac5B or Bac25 (corresponding to about 8 x 10^7 cells) were loaded onto a 12 ml gradient. Centrifugation was carried out for 20 hours at 39000 x g using a Beckman SW40 rotor. 0.5 ml fractions were collected and assayed for activity. The NS5B protein, identified by western blotting, was found to migrate in the density gradients with an unexpectedly high sedimentation coefficient. The viral protein and ribosomes were found to co-sediment in the same gradient fractions. This unique behaviour enabled us to separate the viral protein from the main bulk of cytoplasmic proteins, which remained on the top of the gradient. The RdRp activity assay revealed that the RdRp activity co-sedimented with the NS5B protein. A terminal nucleotidyl transferase activity (TNTase) was also present in these fractions.

EXAMPLE 6

Method for the purification of the HCV TNTase/RdRp from Sf9 cells

Whole cell extracts are made from 1 g of Sf9 cells infected with Bac5B recombinant baculovirus. The frozen cells are thawed on ice in 10 ml of buffer containing 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 10 mM DTT, 50% glycerol (N buffer) supplemented with 1 mM PMSF. Triton X-100 and NaCl are then added to a final concentration of 2% and 500 mM, respectively, in order to promote cell breakage. After the addition of $MgCl_2$ (10 mM) and DNase I (15 µg/ml), the mixture is stirred at room temperature for 30 minutes. The extract is then cleared by ultracentrifugation in a Beckman centrifuge, using a 90 Ti rotor at 40,000 rpm for 30 minutes at 4° C. The cleared extract is diluted with a buffer containing 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 10 mM DTT, 20% glycerol, 0.5% Triton X-100 (LG buffer) in order to adjust the NaCl concentration to 300 mM and incubated batchwise with 5 ml

of DEAE-Sepharose Fast Flow, equilibrated in LG buffer containing 300 mM NaCl. The matrix is then poured into a column and wash d with two volumes of the same buffer. The flow-through and the first wash of the DEAE-Sepharose Fast Flow column is diluted 1:3 with LG buffer and applied onto a Heparin-Sepharose CL6B column (10 ml) equilibrated with LG buffer containing 100 mM NaCl. The Heparin-Sepharose CL6B is washed thoroughly and the bound proteins are eluted with a linear 100 ml gradient, from 100 mM to 1M NaCl in buffer LG. The fractions containing NS5B, as judged by silver- and immuno-staining of SDS-PAGE, are pooled and diluted with LG buffer in order to adjust the NaCl concentration to 50 mM. The diluted fractions are subsequently applied to a Mono Q-FPLC column (1 ml) equilibrated with LG buffer containing 50 mM NaCl. Proteins are eluted with a linear gradient (20 ml) from 50 mM to 1M NaCl in LG buffer. The fractions containing NS5B, as judged by silver- and immuno-staining of SDS-PAGE, are pooled and dialysed against LG buffer containing 100 mM NaCl. After extensive dialysis, the pooled fractions were loaded onto a PoyU-Sepharose CL6B (10 ml) equilibrated with LG buffer containing 100 mM NaCl. The PoyU-Sepharose CL6B was washed thoroughly and the bound proteins were eluted with a linear 100 ml gradient, from 100 mM to 1M NaCl in buffer LG. The fractions containing NS5B, as judged by silver- and immuno-staining of SDS-PAGE, are pooled, dialysed against LG buffer containing 100 mM NaCl and stored in liquid nitrogen prior to activity assay.

Fractions containing the purified protein NS5B were tested for the presence of both activities. The RdRp and TNTase activities were found in the same fractions. These results indicate that both activities, RNA-dependent RNA polymerase and terminal ribonucleotide transferase are the functions of the HCV NS5B protein.

We tested the purified NS5B for terminal nucleotidyl transferase activity with each of the four ribonucleotide

triphosphates at non-saturating substrate concentrations. The results clearly showed that UTP is the preferred TNTase substrate, followed by ATP, CTP and GTP irrespective of the origin of the input RNA.

EXAMPLE 7

Method of assay of recombinant HCV RdRp on a homopolymeric RNA template

Thus far we have described that HCV NS5B possesses an RNA-dependent RNA polymerase activity and that the synthesis of complementary RNA strand is a template-primed reaction. Interestingly, using unfractionated cytoplasmic extracts of Bac5B or Bac25 infected Sf9 cells as a source of RdRp we were not able to observe complementary strand RNA synthesis that utilized an exogenously added oligonucleotide as a primer. We reasoned that this could be due to the abundant ATP-dependent RNA-helicases that would certainly be present in our unfractionated extracts. We therefore wanted to address this question using the purified NS5B.

First of all, we wanted to establish whether the purified NS5B polymerase is capable of synthesizing RNA in a primer-dependent fashion on a homopolymeric RNA template: such a template should not be able to form intramolecular hairpins and therefore we expected that complementary strand RNA synthesis be strictly primer-dependent. We thus measured UMP incorporation dependent on poly(A) template and evaluated both oligo(rU)$_{12}$ and oligo(dT)$_{12-18}$ as primers for the polymerase reaction. Incorporation of radioactive UMP was measured as follows. The standard reaction (10 -100 µl) was carried out in a buffer containing 20 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 25 mM KCl, 1 mM EDTA, 20 U RNasin (Promega), 1 µCi [^{32}P] UTP (400 Ci/mmol, Amersham) or 1 µCi [^{3}H] UTP (55 Ci/mmol, Amersham), 10 µM UTP, and 10 µg/ml poly(A) or poly(A)/oligo(dT)$_{12-18}$. Oligo(U)$_{12}$ (1µg/ml) was added a primer. Poly A and polyA/oligodT_{12-18} were purchased from Pharmacia. Oligo(U)$_{12}$ was obtained from Genset. The final

NS5B enzyme concentration was 10-100 nM. Under these conditions the reaction procedeed linearly for up to 3 h hours. After 2 hours of incubation at 22_, the reaction was stopped by applying the samples to DE81 filters (Whatman), the filters washed thoroughly with 1M Na_2HPO_4/NaH_2PO_4, pH 7.0, rinsed with water, air dried and finally the filter-bound radioactivity was measured in a scintillation ß-counter. Alternatively, the in vitro-synthesized radioactive product was precipitated by 10% trichloroacetic acid with 100 µg of carrier tRNA in 0.2 M sodium pyrophosphate, collected on 0.45-µm Whatman GF/C filters, vacuum dried, and counted in scintilaltion fluid.

Although some [^{32}P]UMP or [^{3}H]UMP ncorporation was detectable even in the absence of a primer and is likely to be due to the terminal nucleotidyl transferase activity associated with our purified NS5B, up to 20% of product incorporation was observed only when oligo(rU)12 was included as primer in the reaction mixture. Unexpectedly, also oligo(dT)12-18 could function as a primer of poly(A)-dependent poly(U) synthesis, albeit with a lower efficiency.

Other template/primers suitable for measuring the RdRp activity of NS5B include poly(C)/oligo(G) or poly(C)/oligo(dG) in the presence of radioactive GTP, poly(G)/oligo(C) or poly(G)/oligo(dC) in the presence of radioactive CTP, poly(U)/oligo(A) or poly(U)/oligo(dA) in the presence of radioactive ATP, poly(I)/oligo(C) or poly(I)/oligo(dC) in the presence of radioactive CTP.

EXAMPLE 8

Method of Expression Of HCV RdRp/TNTase in E. Coli

The plasmid pT7-7(NS5B), described in Figure 2 and Example 8, was constructed in order to allow expression in E. coli of the HCV protein fragment having the sequence reported in SEQ ID NO 1. Such protein fragment contains the RdRp and the TNTase of NS5B, as discussed above. The fragment of HCV cDNA coding for the NS5B

protein was thus cloned downstream of the bacteriophage T7 Ø10 promoter and in frame with the first ATG codon of the phage T7 gene 10 protein, usig methods that are known to the molecular biology practice and described in detail in Example 8. The pT7-7(NS5B) plasmid also contains the gene for the b-lactamase enzyme that can be used as a marker of selection of E. coli cells transformed with plasmid pT7-7(NS5B).

The plasmid pT7-7(NS5B) was then transformed in the E. coli strain BL21(DE53), which is normally employed for high-level expression of genes cloned into expression vectors containing T7 promoter. In this strain of E. coli, the T7 gene polymerase is carried on the bacteriophage 1 DE53, which is integrated into the chromosome of BL21 cells (Studier and Moffatt, Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, (1986), J. Mol. Biol. 189, p. 113-130). Expression from the gene of interest is induced by addition of isopropylthiogalactoside (IPTG) to the growth medium according to a procedure that has been previously described (Studier and Moffatt, 1986). The recombinant NS5B protein fragment containing the RdRp is thus produced in the inclusion bodies of the host cells. Recombinant NS5B protein can be purified from the particulate fraction of E. coli BL21(DE53) extracts and refolded according to procedures that are known in the art (D. R. Thatcher and A. Hichcok, Protein folding in Biotechnology (1994) in "Mechanism of protein folding" R. H. Pain EDITOR, IRL PRESS, p.229-255). Alternatively, the recombinant NS5B protein could be produced as soluble protein by lowering the temperature of the bacterial growth media below 20_ C. The soluble protein could thus be purified from lysates of E. coli substantially as described in Example 5.

EXAMPLE 9

Detailed construction of the plasmids in figures

Selected fragments of the cDNA corresponding to the genome of the HCV-BK isolate (HCVBK) were cloned under the strong polyhedrin promoter of the nuclear polyhedrosis virus and flanked by sequences that allowed homologous recombination in a baculovirus vector.

pBac5Bcontains the HCV-BK sequence comprised between nucleotide 7590 and 9366, and codes for the NS5B protein reported in SEQ ID NO: 1. In order to obtain this plasmid, a cDNA fragment was generated by PCR using synthetic oligonucleotides having the sequences 5'-AAGGATCCATGTCAATGTCCTACACATGGAC-3' (SEQ ID NO: 6) and 5'-AATATTCGAATTCATCGGTTGGGGAGCAGGTAGATG-3' (SEQ ID NO: 7), respectively. The PCR product was then treated with the Klenow DNA polymerase, digested at the 5'-end with *BamHI*, and subsequently cloned between the *BamHI* and *SmaI* sites of the Bluescript SK(+) vector. Subsequently, the cDNA fragment of interest was digested out with the restriction enzymes BamHI and HindIII and religated in the same sites of the pBlueBacIII vector (Invitrogen).

pBac25 is contains the HCV-BK cDNA region comprised between nucleotides 2759 and 9416 of and codes for amino acids 810 to 3010 of the HCV-BK polyprotein (SEQ ID NO: 2). This construct was obtained as follows. First, the 820bp cDNA fragment containing the HCV-BK sequence comprised between nucleotides 2759 and 3578 was obtained from pCD(38-9.4) (Tomei L., Failla,C., Santolini, E., De Francesco, R. and La Monica, N. (1993) NS3 is a Serine Protease Required for Processing of Hepatitis C Virus Polyprotein*J. Virol.*, 67 , 4017-4026) by digestion with *NcoI* and cloned in the *NcoI* site of the pBlueBacIII vector (Invitrogen) yielding a plasmid called pBacNCO.. The cDNA fragment containing the HCV-BK sequence comprised between nucleotides 1959 and 9416 was obtained from pCD(38-9.4) (Tomei et al., 1993) by digestion with *NotI* and *XbaI* and cloned in the same sites of the Bluescript SK(+) vector yielding a plasmid called pBlsNX. The cDNA fragment containing the HCV-BK

sequence comprised between nucleotides 3304 and 9416 was obtained from pBlsNX by digestion with *SacII*and *HindIII* and cloned in the same sites of the pBlsNX plasmid, yielding the pBac25 plasmid.

pT7-7(DCoH) contains the entire coding region (316 nucleotides) of the rat dimerization cofactor of hepatocyte nuclear factor-laå (DCoH; Mendel, D.B., Khavari, P.A., Conley, P.B., Graves, M.K., Hansen, L.P., Admon, A. and Crabtree, G.R. (1991) Characterization of a Cofactor that Regulates Dimerization of a Mammalian Homeodomain Protein, *Science* 254, 1762-1767; GenBank accession number: M83740). The cDNA fragment corresponding to the coding sequence for rat DCoH was amplified by PCR using the synthetic oligonucleotide Dpr1 and Dpr2 that have the sequence TGGCTGGCAAGGCACACAGGCT (SEQ ID NO: 8) and AGGCAGGGTAGATCTATGTC (SEQ ID NO: 9), respectively. The cDNA fragment thus obtained was cloned into the *SmaI* restriction site of the E. coli expression vector pT7-7. The pT7-7 expression vector is ea derivative of pBR322 that contains, in addition to the ß-lactamase gene and the *Col* E1 orifgin of replication, the T7 polymerase promoter Ø10 and the translational start site for the T7 gene 10 protein (Tabor S. and Richerdson C. C.(1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes, *Proc. Natl. Acad. Sci. USA* 82, 1074-1078).

pT7-7(NS5B) contains the HCV sequence from nucleotide 7590 to nucleotide 9366, and codes for the NS5B protein reported in SEQ ID NO: 1.

In order to obtain this plasmid, a cDNA fragment was generated by PCR using synthetic oligonucleotides having the sequences 5'-TCAATGTCCTACACATGGAC-3' (SEQ ID NO: 10) and 5'-GATCTCTAGATCATCGGTTGGGGGAGGAGGTAGATGCC-3' (SEQ ID NO: 11), respectively. The PCR product was then treated with the Klenow DNA polymerase, and subsequently ligated in the E. coli expression vector pT7-7 after linearizing

it with *EcoRI* and blunting its estremities with the Klenow DNA polymerase. Alternatively, cDNA fragment was generated by PCR using synthetic oligonucleotides having the sequences 5'- TGTCAATGTCCTACACATGG-3' (SEQ ID NO: 13) and 5'-AATATTCGAATTCATCGGTTGGGGAGCAGGTAGATG-3' (SEQ ID NO: 14), respectively. The PCR product was then treated with the Klenow DNA polymerase, and subsequently ligated in the E. coli expression vector pT7-7 after linearizing it with *NdeI* and blunting its estremities with the Klenow DNA polymerase.

SEQUENCE LISTING

GENERAL INFORMATION

(i) APPLICANT: ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI S.p.A.

(ii) TITLE OF INVENTION: METHOD FOR REPRODUCING IN VITRO THE RNA-DEPENDENT RNA POLYMERASE AND TERMINAL NUCLEOTIDYL TRANSFERASE ACTIVITIES ENCODED BY HEPATITIS C VIRUS (HCV)

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Societa Italiana Brevetti
(B) STREET: Piazza di Pietra, 39
(C) CITY: Rome
(D) COUNTRY: Italy
(E) POSTAL CODE: 1-00186

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk 3.5" 1.44 MBYTES
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS Rev.6.22
(D) SOFTWARE: Microsoft Word 6.0

(viii) ATTORNEY INFORMATION
(A) NAME: DI CERBO, Mario (Dr.)
(C) REFERENCE: RM/X88530/PCT-DC

(ix) TELECOMMUNICATION INFORMATION
(A) TELEPHONE: 06/6785941
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(C) TELEX: 612287 ROPAT

(1) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 591 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: C-terminal fragment

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hepatitis C Virus

(C) ISOLATE : BK

(vii) IMMEDIATE SOURCE: cDNA clone pCD(38-9.4) described by Tomei et al. 1993

(ix) FEATURE:

(A) NAME: NS5B Non-structural polyprotein

(C) IDENTIFICATION METHOD: Experimentally

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser Met Ser Tyr Thr Trp Thr Gly Ala Leu Ile Thr Pro Cys Ala Ala
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Glu Glu Ser Lys Leu Pro Ile Asn Ala Leu Ser Asn Ser Leu Leu Arg
20 25 30

His His Asn Met Val Tyr Ala Thr Thr Ser Arg Ser Ala Gly Leu Arg
35 40 45

Gln Lys Lys Val Thr Phe Asp Arg Leu Gln Val Leu Asp Asp His Tyr
50 55 60

Arg Asp Val Leu Lys Glu Met Lys Ala Lys Ala Ser Thr Val Lys Ala
65 70 75 80

Lys Leu Leu Ser Val Glu Glu Ala Cys Lys Leu Thr Pro Pro His Ser
85 90 95

Ala Lys Ser Lys Phe Gly Tyr Gly Ala Lys Asp Val Arg Asn Leu Ser
100 105 110

Ser Lys Ala Val Asn His Ile His Ser Val Trp Lys Asp Leu Leu Glu
115 120 125

Asp Thr Val Thr Pro Ile Asp Thr Thr Ile Met Ala Lys Asn Glu Val
130 135 140

Phe Cys Val Gln Pro Glu Lys Gly Gly Arg Lys Pro Ala Arg Leu Ile
145 150 155 160

Val Phe Pro Asp Leu Gly Val Arg Val Cys Glu Lys Met Ala Leu Tyr
165 170 175

Asp Val Val Ser Thr Leu Pro Gln Val Val Met Gly Ser Ser Tyr Gly
180 185 190

Phe Gln Tyr Ser Pro Gly Gln Arg Val Glu Phe Leu Val Asn Thr Trp

195 200 205

Lys Ser Lys Lys Asn Pro Met Gly Phe Ser Tyr Asp Thr Arg Cys Phe
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Asp Ser Thr Val Thr Glu Asn Asp Ile Arg Val Glu Glu Ser Ile Tyr
225 230 235 240

Gln Cys Cys Asp Leu Ala Pro Glu Ala Arg Gln Ala Ile Lys Ser Leu
245 250 255

Thr Glu Arg Leu Tyr Ile Gly Gly Pro Leu Thr Asn Ser Lys Gly Gln
260 265 270

Asn Cys Gly Tyr Arg Arg Cys Arg Ala Ser Gly Val Leu Thr Thr Ser
275 280 285

Cys Gly Asn Thr Leu Thr Cys Tyr Leu Lys Ala Ser Ala Ala Cys Arg
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Ala Ala Lys Leu Gln Asp Cys Thr Met Leu Val Asn Gly Asp Asp Leu
305 310 315 320

Val Val Ile Cys Glu Ser Ala Gly Thr Gln Glu Asp Ala Ala Ser Leu
325 330 335

Arg Val Phe Thr Glu Ala Met Thr Arg Tyr Ser Ala Pro Pro Gly Asp
340 345 350

Pro Pro Gln Pro Glu Tyr Asp Leu Glu Leu Ile Thr Ser Cys Ser Ser
355 360 365

Asn Val Ser Val Ala His Asp Ala Ser Gly Lys Arg Val Tyr Tyr Leu
370 375 380

Thr Arg Asp Pro Thr Thr Pro Leu Ala Arg Ala Ala Trp Glu Thr Ala
385 390 395 400

Arg His Thr Pro Val Asn Ser Trp Leu Gly Asn Ile Ile Met Tyr Ala
405 410 415

Pro Thr Leu Trp Ala Arg Met Ile Leu Met Thr His Phe Phe Ser Ile
420 425 430

Leu Leu Ala Gln Glu Gln Leu Glu Lys Ala Leu Asp Cys Gln Ile Tyr
435 440 445

Gly Ala Cys Tyr Ser Ile Glu Pro Leu Asp Leu Pro Gln Ile Ile Glu
450 455 460

Arg Leu His Gly Leu Ser Ala Phe Ser Leu His Ser Tyr Ser Pro Gly
465 470 475 480

Glu Ile Asn Arg Val Ala Ser Cys Leu Arg Lys Leu Gly Val Pro Pro

485 490 495

Leu Arg Val Trp Arg His Arg Ala Arg Ser Val Arg Ala Arg Leu Leu
500 505 510

Ser Gln Gly Gly Arg Ala Ala Thr Cys Gly Lys Tyr Leu Phe Asn Trp
515 520 525

Ala Val Lys Thr Lys Leu Lys Leu Thr Pro Ile Pro Ala Ala Ser Arg
530 535 540

Leu Asp Leu Ser Gly Trp Phe Val Ala Gly Tyr Ser Gly Gly Asp Ile
545 550 555 560

Tyr His Ser Leu Ser Arg Ala Arg Pro Arg Trp Phe Met Leu Cys Leu
565 570 575

Leu Leu Leu Ser Val Gly Val Gly Ile Tyr Leu Leu Pro Asn Arg
580 585 590

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 2201 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: C-terminal fragment

(vii) IMMEDIATE SOURCE: cDNA clone pCD(38-9.4) described by Tomei et al. 1993

(ix) FEATURE:

(A) NAME: NS2-NS5B Nonstructural Protein Precursor

(C) IDENTIFICATION METHOD: Experimentally

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Asp Arg Glu Met Ala Ala Ser Cys Gly Gly Ala Val Phe Val Gly
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Leu Val Leu Leu Thr Leu Ser Pro Tyr Tyr Lys Val Phe Leu Ala Arg
20 25 30

Leu Ile Trp Trp Leu Gln Tyr Phe Thr Thr Arg Ala Glu Ala Asp Leu

35 40 45

His Val Trp Ile Pro Pro Leu Asn Ala Arg Gly Gly Arg Asp Ala Ile

50 55 60

Ile Leu Leu Met Cys Ala Val His Pro Glu Leu Ile Phe Asp Ile Thr

65 70 75 80

Lys Leu Leu Ile Ala Ile Leu Gly Pro Leu Met Val Leu Gln Ala Gly

85 90 95

Ile Thr Arg Val Pro Tyr Phe Val Arg Ala Gln Gly Leu Ile His Ala

100 105 110

Cys Met Leu Val Arg Lys Val Ala Gly Gly His Tyr Val Gln Met Ala

115 120 125

Phe Met Lys Leu Gly Ala Leu Thr Gly Thr Tyr Ile Tyr Asn His Leu

130 135 140

Thr Pro Leu Arg Asp Trp Pro Arg Ala Gly Leu Arg Asp Leu Ala Val

145 150 155 160

Ala Val Glu Pro Val Val Phe Ser Asp Met Glu Thr Lys Ile Ile Thr

165 170 175

Trp Gly Ala Asp Thr Ala Ala Cys Gly Asp Ile Ile Leu Gly Leu Pro

180 185 190

Val Ser Ala Arg Arg Gly Lys Glu Ile Leu Leu Gly Pro Ala Asp Ser

195 200 205

Leu Glu Gly Arg Gly Leu Arg Leu Leu Ala Pro Ile Thr Ala Tyr Ser

210 215 220

Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile Thr Ser Leu Thr Gly

225 230 235 240

Arg Asp Lys Asn Gln Val Glu Gly Glu Val Gln Val Val Ser Thr Ala

245 250 255

Thr Gln Ser Phe Leu Ala Thr Cys Val Asn Gly Val Cys Trp Thr Val

260 265 270

Tyr His Gly Ala Gly Ser Lys Thr Leu Ala Ala Pro Lys Gly Pro Ile

275 280 285

Thr Gln Met Tyr Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Lys

290 295 300

Pro Pro Gly Ala Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp

305 310 315 320

Leu Tyr Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg

325 330 335

Gly Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Val Ser Tyr Leu
340 345 350

Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Phe Gly His Ala Val
355 360 365

Gly Ile Phe Arg Ala Ala Val Cys Thr Arg Gly Val Ala Lys Ala Val
370 375 380

Asp Phe Val Pro Val Glu Ser Met Glu Thr Thr Met Arg Ser Pro Val
385 390 395 400

Phe Thr Asp Asn Ser Ser Pro Pro Ala Val Pro Gln Ser Phe Gln Val
405 410 415

Ala His Leu His Ala Pro Thr Gly Ser Gly Lys Ser Thr Lys Val Pro
420 425 430

Ala Ala Tyr Ala Ala Gln Gly Tyr Lys Val Leu Val Leu Asn Pro Ser
435 440 445

Val Ala Ala Thr Leu Gly Phe Gly Ala Tyr Met Ser Lys Ala His Gly
450 455 460

Ile Asp Pro Asn Ile Arg Thr Gly Val Arg Thr Ile Thr Thr Gly Ala
465 470 475 480

Pro Val Thr Tyr Ser Thr Tyr Gly Lys Phe Leu Ala Asp Gly Gly Cys
485 490 495

Ser Gly Gly Ala Tyr Asp Ile Ile Ile Cys Asp Glu Cys His Ser Thr
500 505 510

Asp Ser Thr Thr Ile Leu Gly Ile Gly Thr Val Leu Asp Gln Ala Glu
515 520 525

Thr Ala Gly Ala Arg Leu Val Val Leu Ala Thr Ala Thr Pro Pro Gly
530 535 540

Ser Val Thr Val Pro His Pro Asn Ile Glu Glu Val Ala Leu Ser Asn
545 550 555 560

Thr Gly Glu Ile Pro Phe Tyr Gly Lys Ala Ile Pro Ile Glu Ala Ile
565 570 575

Arg Gly Gly Arg His Leu Ile Phe Cys His Ser Lys Lys Lys Cys Asp
580 585 590

Glu Leu Ala Ala Lys Leu Ser Gly Leu Gly Ile Asn Ala Val Ala Tyr
595 600 605

Tyr Arg Gly Leu Asp Val Ser Val Ile Pro Thr Ile Gly Asp Val Val
610 615 620

```
Val Val Ala Thr Asp Ala Leu Met Thr Gly Tyr Thr Gly Asp Phe Asp
625                 630                 635                 640

Ser Val Ile Asp Cys Asn Thr Cys Val Thr Gln Thr Val Asp Phe Ser
                645                 650                 655
Leu Asp Pro Thr Phe Thr Ile Glu Thr Thr Thr Val Pro Gln Asp Ala
            660                 665                 670
Val Ser Arg Ser Gln Arg Arg Gly Arg Thr Gly Arg Gly Arg Arg Gly
        675                 680                 685
Ile Tyr Arg Phe Val Thr Pro Gly Glu Arg Pro Ser Gly Met Phe Asp
    690                 695                 700
Ser Ser Val Leu Cys Glu Cys Tyr Asp Ala Gly Cys Ala Trp Tyr Glu
705                 710                 715                 720
Leu Thr Pro Ala Glu Thr Ser Val Arg Leu Arg Ala Tyr Leu Asn Thr
                725                 730                 735
Pro Gly Leu Pro Val Cys Gln Asp His Leu Glu Phe Trp Glu Ser Val
            740                 745                 750
Phe Thr Gly Leu Thr His Ile Asp Ala His Phe Leu Ser Gln Thr Lys
        755                 760                 765
Gln Ala Gly Asp Asn Phe Pro Tyr Leu Val Ala Tyr Gln Ala Thr Val
    770                 775                 780
Cys Ala Arg Ala Gln Ala Pro Pro Pro Ser Trp Asp Gln Met Trp Lys
785                 790                 795                 800
Cys Leu Ile Arg Leu Lys Pro Thr Leu His Gly Pro Thr Pro Leu Leu
                805                 810                 815
Tyr Arg Leu Gly Ala Val Gln Asn Glu Val Thr Leu Thr His Pro Ile
            820                 825                 830
Thr Lys Tyr Ile Met Ala Cys Met Ser Ala Asp Leu Glu Val Val Thr
        835                 840                 845
Ser Thr Trp Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr
    850                 855                 860
Cys Leu Thr Thr Gly Ser Val Val Ile Val Gly Arg Ile Ile Leu Ser
865                 870                 875                 880
Gly Arg Pro Ala Ile Val Pro Asp Arg Glu Leu Leu Tyr Gln Glu Phe
                885                 890                 895
Asp Glu Met Glu Glu Cys Ala Ser His Leu Pro Tyr Ile Glu Gln Gly
            900                 905                 910
```

Met Gln Leu Ala Glu Gln Phe Lys Gln Lys Ala Leu Gly Leu Leu Gln
915 920 925

Thr Ala Thr Lys Gln Ala Glu Ala Ala Ala Pro Val Val Glu Ser Lys
930 935 940

Trp Arg Ala Leu Glu Thr Phe Trp Ala Lys His Met Trp Asn Phe Ile
945 950 955 960

Ser Gly Ile Gln Tyr Leu Ala Gly Leu Ser Thr Leu Pro Gly Asn Pro
965 970 975

Ala Ile Ala Ser Leu Met Ala Phe Thr Ala Ser Ile Thr Ser Pro Leu
980 985 990

Thr Thr Gln Ser Thr Leu Leu Phe Asn Ile Leu Gly Gly Trp Val Ala
995 1000 1005

Ala Gln Leu Ala Pro Pro Ser Ala Ala Ser Ala Phe Val Gly Ala Gly
1010 1015 1020

Ile Ala Gly Ala Ala Val Gly Ser Ile Gly Leu Gly Lys Val Leu Val
1025 1030 1035 1040

Asp Ile Leu Ala Gly Tyr Gly Ala Gly Val Ala Gly Ala Leu Val Ala
1045 1050 1055

Phe Lys Val Met Ser Gly Glu Met Pro Ser Thr Glu Asp Leu Val Asn
1060 1065 1070

Leu Leu Pro Ala Ile Leu Ser Pro Gly Ala Leu Val Val Gly Val Val
1075 1080 1085

Cys Ala Ala Ile Leu Arg Arg His Val Gly Pro Gly Glu Gly Ala Val
1090 1095 1100

Gln Trp Met Asn Arg Leu Ile Ala Phe Ala Ser Arg Gly Asn His Val
1105 1110 1115 1120

Ser Pro Thr His Tyr Val Pro Glu Ser Asp Ala Ala Ala Arg Val Thr
1125 1130 1135

Gln Ile Leu Ser Ser Leu Thr Ile Thr Gln Leu Leu Lys Arg Leu His
1140 1145 1150

Gln Trp Ile Asn Glu Asp Cys Ser Thr Pro Cys Ser Gly Ser Trp Leu
1155 1160 1165

Arg Asp Val Trp Asp Trp Ile Cys Thr Val Leu Thr Asp Phe Lys Thr
1170 1175 1180

Trp Leu Gln Ser Lys Leu Leu Pro Gln Leu Pro Gly Val Pro Phe Phe
1185 1190 1195 1200

Ser Cys Gln Arg Gly Tyr Lys Gly Val Trp Arg Gly Asp Gly Ile Met
1205 1210 1215

Gln Thr Thr Cys Pro Cys Gly Ala Gln Ile Thr Gly His Val Lys Asn
1220 1225 1230

Gly Ser Met Arg Ile Val Gly Pro Lys Thr Cys Ser Asn Thr Trp His
1235 1240 1245

Gly Thr Phe Pro Ile Asn Ala Tyr Thr Thr Gly Pro Cys Thr Pro Ser
1250 1255 1260

Pro Ala Pro Asn Tyr Ser Arg Ala Leu Trp Arg Val Ala Ala Glu Glu
1265 1270 1275 1280

Tyr Val Glu Val Thr Arg Val Gly Asp Phe His Tyr Val Thr Gly Met
1285 1290 1295

Thr Thr Asp Asn Val Lys Cys Pro Cys Gln Val Pro Ala Pro Glu Phe
1300 1305 1310

Phe Ser Glu Val Asp Gly Val Arg Leu His Arg Tyr Ala Pro Ala Cys
1315 1320 1325

Arg Pro Leu Leu Arg Glu Glu Val Thr Phe Gln Val Gly Leu Asn Gln
1330 1335 1340

Tyr Leu Val Gly Ser Gln Leu Pro Cys Glu Pro Glu Pro Asp Val Ala
1345 1350 1355 1360

Val Leu Thr Ser Met Leu Thr Asp Pro Ser His Ile Thr Ala Glu Thr
1365 1370 1375

Ala Lys Arg Arg Leu Ala Arg Gly Ser Pro Pro Ser Leu Ala Ser Ser
1380 1385 1390

Ser Ala Ser Gln Leu Ser Ala Pro Ser Leu Lys Ala Thr Cys Thr Thr
1395 1400 1405

His His Val Ser Pro Asp Ala Asp Leu Ile Glu Ala Asn Leu Leu Trp
1410 1415 1420

Arg Gln Glu Met Gly Gly Asn Ile Thr Arg Val Glu Ser Glu Asn Lys
1425 1430 1435 1440

Val Val Val Leu Asp Ser Phe Asp Pro Leu Arg Ala Glu Glu Asp Glu
1445 1450 1455

Arg Glu Val Ser Val Pro Ala Glu Ile Leu Arg Lys Ser Lys Lys Phe
1460 1465 1470

Pro Ala Ala Met Pro Ile Trp Ala Arg Pro Asp Tyr Asn Pro Pro Leu
1475 1480 1485

Leu Glu Ser Trp Lys Asp Pro Asp Tyr Val Pro Pro Val Val His Gly
1490 1495 1500

Cys Pro Leu Pro Pro Ile Lys Ala Pro Pro Ile Pro Pro Pro Arg Arg
1505 1510 1515 1520
Lys Arg Thr Val Val Leu Thr Glu Ser Ser Val Ser Ser Ala Leu Ala
1525 1530 1535
Glu Leu Ala Thr Lys Thr Phe Gly Ser Ser Glu Ser Ser Ala Val Asp
1540 1545 1550
Ser Gly Thr Ala Thr Ala Leu Pro Asp Gln Ala Ser Asp Asp Gly Asp
1555 1560 1565
Lys Gly Ser Asp Val Glu Ser Tyr Ser Ser Met Pro Pro Leu Glu Gly
1570 1575 1580
Glu Pro Gly Asp Pro Asp Leu Ser Asp Gly Ser Trp Ser Thr Val Ser
1585 1590 1595 1600
Glu Glu Ala Ser Glu Asp Val Val Cys Cys Ser Met Ser Tyr Thr Trp
1605 1610 1615
Thr Gly Ala Leu Ile Thr Pro Cys Ala Ala Glu Glu Ser Lys Leu Pro
1620 1625 1630
Ile Asn Ala Leu Ser Asn Ser Leu Leu Arg His His Asn Met Val Tyr
1635 1640 1645
Ala Thr Thr Ser Arg Ser Ala Gly Leu Arg Gln Lys Lys Val Thr Phe
1650 1655 1660
Asp Arg Leu Gln Val Leu Asp Asp His Tyr Arg Asp Val Leu Lys Glu
1665 1670 1675 1680
Met Lys Ala Lys Ala Ser Thr Val Lys Ala Lys Leu Leu Ser Val Glu
1685 1690 1695
Glu Ala Cys Lys Leu Thr Pro Pro His Ser Ala Lys Ser Lys Phe Gly
1700 1705 1710
Tyr Gly Ala Lys Asp Val Arg Asn Leu Ser Ser Lys Ala Val Asn His
1715 1720 1725
Ile His Ser Val Trp Lys Asp Leu Leu Glu Asp Thr Val Thr Pro Ile
1730 1735 1740
Asp Thr Thr Ile Met Ala Lys Asn Glu Val Phe Cys Val Gln Pro Glu
1745 1750 1755 1760
Lys Gly Gly Arg Lys Pro Ala Arg Leu Ile Val Phe Pro Asp Leu Gly
1765 1770 1775

Val Arg Val Cys Glu Lys Met Ala Leu Tyr Asp Val Val Ser Thr Leu
1780 1785 1790

Pro Gln Val Val Met Gly Ser Ser Tyr Gly Phe Gln Tyr Ser Pro Gly
1795 1800 1805

Gln Arg Val Glu Phe Leu Val Asn Thr Trp Lys Ser Lys Lys Asn Pro
1810 1815 1820

Met Gly Phe Ser Tyr Asp Thr Arg Cys Phe Asp Ser Thr Val Thr Glu
1825 1830 1835 1840

Asn Asp Ile Arg Val Glu Glu Ser Ile Tyr Gln Cys Cys Asp Leu Ala
1845 1850 1855

Pro Glu Ala Arg Gln Ala Ile Lys Ser Leu Thr Glu Arg Leu Tyr Ile
1860 1865 1870

Gly Gly Pro Leu Thr Asn Ser Lys Gly Gln Asn Cys Gly Tyr Arg Arg
1875 1880 1885

Cys Arg Ala Ser Gly Val Leu Thr Thr Ser Cys Gly Asn Thr Leu Thr
1890 1895 1900

Cys Tyr Leu Lys Ala Ser Ala Ala Cys Arg Ala Ala Lys Leu Gln Asp
1905 1910 1915 1920

Cys Thr Met Leu Val Asn Gly Asp Asp Leu Val Val Ile Cys Glu Ser
1925 1930 1935

Ala Gly Thr Gln Glu Asp Ala Ala Ser Leu Arg Val Phe Thr Glu Ala
1940 1945 1950

Met Thr Arg Tyr Ser Ala Pro Pro Gly Asp Pro Pro Gln Pro Glu Tyr
1955 1960 1965

Asp Leu Glu Leu Ile Thr Ser Cys Ser Ser Asn Val Ser Val Ala His
1970 1975 1980

Asp Ala Ser Gly Lys Arg Val Tyr Tyr Leu Thr Arg Asp Pro Thr Thr
1985 1990 1995 2000

Pro Leu Ala Arg Ala Ala Trp Glu Thr Ala Arg His Thr Pro Val Asn
2005 2010 2015

Ser Trp Leu Gly Asn Ile Ile Met Tyr Ala Pro Thr Leu Trp Ala Arg
2020 2025 2030

Met Ile Leu Met Thr His Phe Phe Ser Ile Leu Leu Ala Gln Glu Gln
2035 2040 2045

Leu Glu Lys Ala Leu Asp Cys Gln Ile Tyr Gly Ala Cys Tyr Ser Ile
2050 2055 2060

Glu Pro Leu Asp Leu Pro Gln Ile Ile Glu Arg Leu His Gly Leu Ser
2065 2070 2075 2080

Ala Phe Ser Leu His Ser Tyr Ser Pro Gly Glu Ile Asn Arg Val Ala
2085 2090 2095

Ser Cys Leu Arg Lys Leu Gly Val Pro Pro Leu Arg Val Trp Arg His
2100 2105 2110

Arg Ala Arg Ser Val Arg Ala Arg Leu Leu Ser Gln Gly Gly Arg Ala
2115 2120 2125

Ala Thr Cys Gly Lys Tyr Leu Phe Asn Trp Ala Val Lys Thr Lys Leu
2130 2135 2140

Lys Leu Thr Pro Ile Pro Ala Ala Ser Arg Leu Asp Leu Ser Gly Trp
2145 2150 2155 2160

Phe Val Ala Gly Tyr Ser Gly Gly Asp Ile Tyr His Ser Leu Ser Arg
2165 2170 2175

Ala Arg Pro Arg Trp Phe Met Leu Cys Leu Leu Leu Leu Ser Val Gly
2180 2185 2190

Val Gly Ile Tyr Leu Leu Pro Asn Arg
2195 2200

(3) INFORMATION FOR SEQ ID NO: 3

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 26 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: oligo a

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

GCCGAGATGC CATCTTCAAA CAGTTC 26

(4) INFORMATION FOR SEQ ID NO: 4

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: oligo b

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

GTGTACAACA AGGTCCATAT CACC 24

(5) INFORMATION FOR SEQ ID NO: 5

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: oligo c

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

GGTCTTTCTG AACGGGATAT AAAC 24

(6) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 31 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: 5'-5B

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6

AAGGATCCAT GTCAATGTCC TACACATGGA C 31

(7) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 36 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: 3'-5B

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

AATATTCGAA TTCATCGGTT GGGGAGCAGG TAGATG 36

(8) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 22 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: Dpr1

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

TGGCTGGCAA GGCACACAGG CT 22

(9) INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: Dpr2

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

AGGCAGGGTA GATCTATGTC 20

(10) INFORMATION FOR SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: NS5B-5'(1)

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

TCAATGTCCT ACACATGGAC 20

(11) INFORMATION FOR SEQ ID NO: 11

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: HCVA-13
(C) IDENTIFICATION METHOD: Polyacrylamide gel
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

GATCTCTAGA TCATCGGTTG GGGGAGGAGG TAGATGCC 38

(12) INFORMATION FOR SEQ ID NO: 12
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 399 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: mRNA
(iii) HYPOTHETICAL: No
(iv) ANTISENSE: No
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rattus Norvegicus
(B) STRAIN : Sprague-Dawley
(vii) IMMEDIATE SOURCE: pT7-7(DCoH)
(ix) FEATURE:
(A) NAME: D-RNA
(C) IDENTIFICATION METHOD: Polyacrylamide gel
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

GGGAGACCAC AACGGUUUCC CUCUAGAAAU AAUUUUGUUU AACUUUAAGA AGGAGAUAUA 60
CAUAUGGCUA GAAUUCGCGC CCUGGCUGGC AAGGCACACA GGCUGAGUGC UGAGGAACGG 120
GACCAGCUGC UGCCAAACCU GCGGGCCGUG GGGUGGAAUG AACUGGAAGG CCGAGAUGCC 180
AUCUUCAAAC AGUUCCAUUU UAAAGACUUC AACAGGGCUU UUGGCUUCAU GACAAGAGUC 240
GCCCUGCAGG CUGAAAAGCU GGACCACCAU CCCGAGUGGU UUAACGUGUA CAACAAGGUC 300
CAUAUCACCU UGAGCACCCA CGAAUGUGCC GGUCUUUCUG AACGGGAUAU AAACCUGGCC 360
AGCUUCAUCG AACAAGUUGC CGUGUCUAUG ACAUAGAUC 399

(13) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: NS5B-up

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

TGTCAATGTC CTACACATGG 20

(14) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(vii) IMMEDIATE SOURCE: oligonucleotide synthesizer

(ix) FEATURE:

(A) NAME: 3'-5B

(C) IDENTIFICATION METHOD: Polyacrylamide gel

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

AATATTCGAA TTCATCGGTT GGGGAGCAGG TAGATG 36

CLAIMS

1. A method for reproducing in vitro the RNA-dependent RNA polymerase activity or the terminal nucleotidyl transferase activity encoded by hepatitis C virus, characterized in that sequences containing NS5B (SEQ ID NO: 1) are used in the reaction mixture.

2. The method for reproducing in vitro the RNA-dependent RNA polymerase activity encoded by HCV according to claim 1, in which NS5B is incorporated in the reaction mixture as NS2-NS5B precursor, said precursor generating, by means of multiple proteolytic events that occur in the overproducing organism, an enzymatically active form of NS5B.

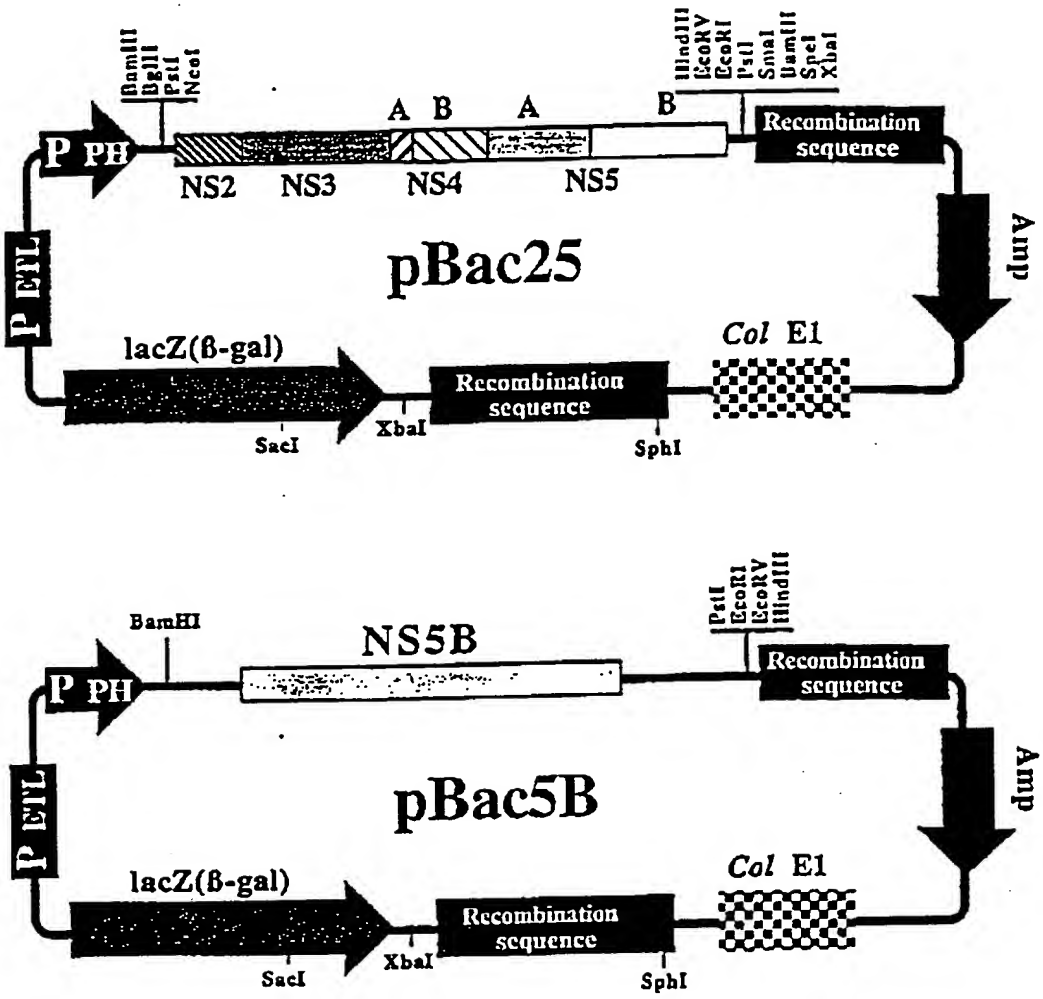
3. The method for reproducing in vitro the terminal nucleotidyl transferase activity encoded by HCV according to claim 1, in which NS5B is incorporated in the reaction mixture as NS2-NS5B precursor, said precursor generating, by means of multiple proteolytic events that occur in the overproducing organism, an enzymatically active form of NS5B.

4. A composition of matter, characterized in that it contains NS5B sequences according to claims 1 to 3.

5. A composition of matter according to claim 4, comprising the proteins whose sequences are described in SEQ ID NO: 1, in sequences contained therein or derived therefrom.

6. Use of the compositions of matter according to claims 4 and 5 to set up an enzymatic test capable of selecting, for therapeutic purposes, compounds that inhibit the enzymatic activity associated with NS5B.

7. Method for reproducing in vitro the RNA-dependent RNA polymerase and terminal nucleotidyl transferase activities of NS5B, compositions of matter and use of said compositions of matter to set up an enzymatic test capable of selecting, for therapeutic purposes, compounds that inhibit the enzymatic activities associated with NS5B, according to the above description, examples and claims.



P ETL = promoter of the gene coding for the PCNA protein

P PH = promoter of the polyhedrin gene

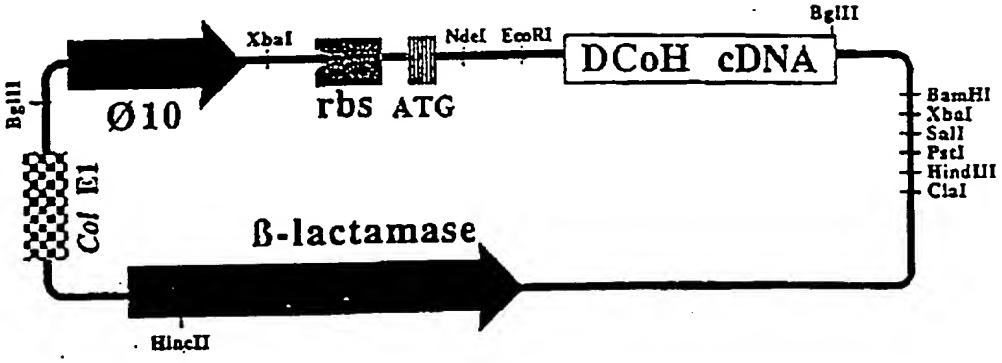
Amp = gene coding for the ß-lactamase enzyme (ampicillin resistence)

LacZ (ß-gal) = gene coding for the ß-galactosidase enzyme

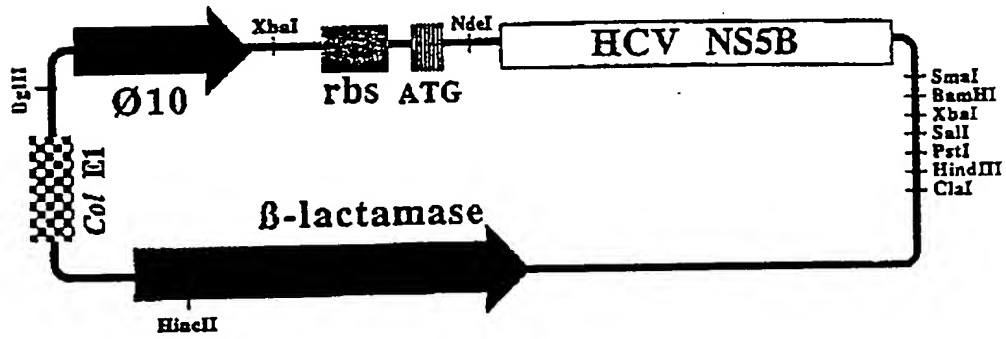
Col E1 = pBR322 replication origin

FIG. 1

pT7-7(DCoH)



pT7-7(NS5B)



Ø10 = bacteriophage T7 Ø10 promoter

rbs = Shine-Dalgarno ribosome binding site

ATG = translation initiation site of the protein coded by the bacteriophage T7 gene 10

ß-lactamase = gene coding for the ß-lactamase enzyme (ampicillin resistance)

Col E1 = pBR322 repliation origin

FIG.2

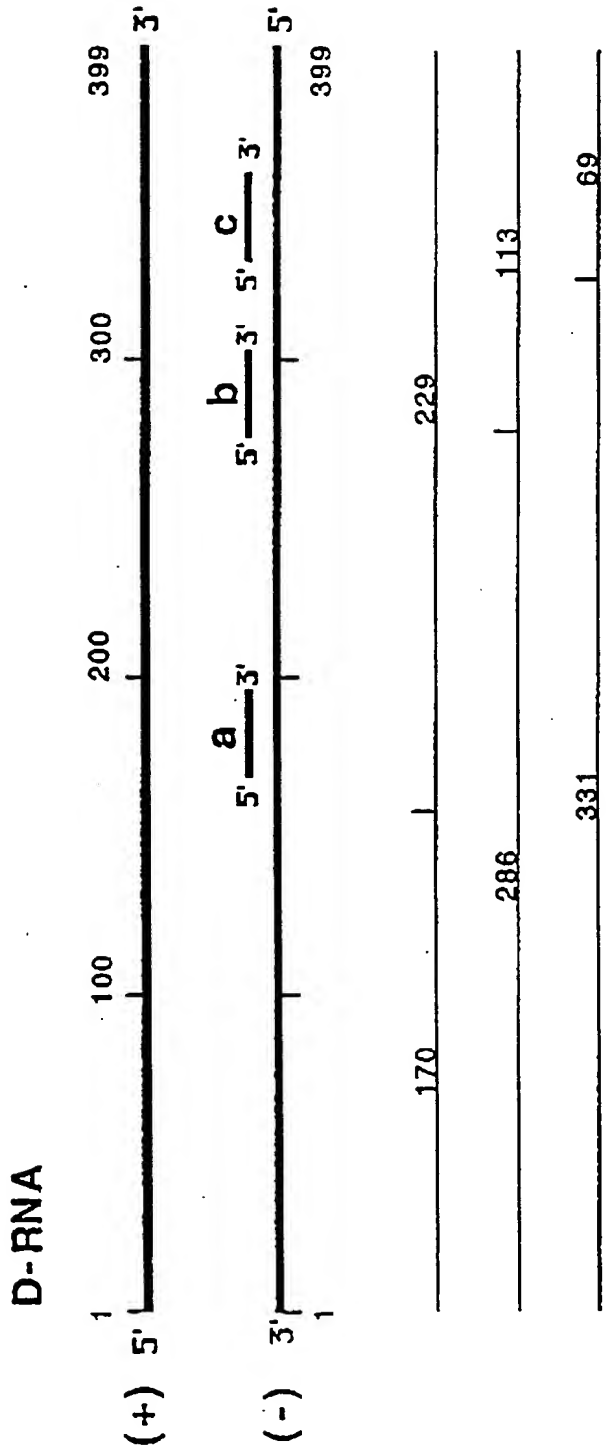


FIG.3

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/IT 96/00106

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N9/12 C12Q1/48 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 463 848 (UNIV OSAKA RES FOUND) 2 January 1992	4,5
Y	see page 3, line 45 - line 50 see page 11, line 7 see page 19, line 39 - line 46 see page 21, line 1 - page 29, line 42 see page 50, line 26 - page 53, line 25 see claims 1-21; figure 1	1,2,6,7

X	EP,A,0 464 287 (UNIV OSAKA RES FOUND) 8 January 1992	4,5
Y	see page 11, line 13 - page 16, line 45; claims 1-31	1,2,6,7

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 1996	03.09.96

Name and mailing address of the ISA	Authorized officer
European Patent ffice, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. VIROLOGY, vol. 67, no. 7, July 1993, AM.SOC.MICROBIOL.,WASHINGTON,US, pages 4017-4026, XP000601449 L. TOMEI ET AL.: "NS3 is a serine protease required for processing of hepatitis C virus polyprotein" cited in the application	4,5
Y	see page 4017, right-hand column, line 18 - line 22 see page 4018, right-hand column, line 35 - page 4019, left-hand column, line 2 see page 4021, left-hand column, line 30 - line 35	1,2,6,7
Y	WO,A,94 05809 (FOX CHASE CANCER CENTER ;SEEGER CHRISTOPH (US)) 17 March 1994 see the whole document	1,2,6,7
A	J. VIROLOGY, vol. 68, no. 8, August 1994, AM.SOC.MICROBIOL.,WASHINGTON,US, pages 5045-5055, XP002012001 R. BARTENSCHLAGER ET AL.: "Kinetic and structural analysis of hepatitis C virus polyprotein processing" see the whole document	1-7
A	J. VIROLOGY, vol. 68, no. 12, December 1994, AM.SOC.MICROBIOL.,WASHINGTON,US, pages 8147-8157, XP002012002 C LIN ET AL.: "Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics" see the whole document	1-7
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, no. 6, 1 March 1990, pages 2057-2061, XP000310581 MILLER R H ET AL: "HEPATITIS C VIRUS SHARES AMINO ACID SEQUENCE SIMILARITY WITH PESTIVIRUSES AND FLAVIVIRUSES AS WELL AS MEMBERS OF TWO PLANT VIRUS SUPERGROUPS" see the whole document	1-7
P,X	WO,A,95 22985 (ISTITUTO DI RICERCHE DI BIOLOG ;FRANCESCO RAFFAELE DE (IT); FAILLA) 31 August 1995	4,5
1 P,Y	see the whole document	1,2,6,7

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INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/IT 96/00106

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL 15 (1). 1996. 12-22. ISSN: 0261-4189, January 1996, XP002012003 BEHRENS S-E ET AL: "Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus." see the whole document	1-7

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/IT 96/00106

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0463848	02-01-92	AU-B-	652941	15-09-94
		AU-B-	7925691	02-01-92
		CA-A-	2045326	26-12-91
		JP-A-	7258290	09-10-95
		CN-A-	1059758	25-03-92
		AU-B-	650634	30-06-94
		AU-B-	6860891	02-01-92
		CA-A-	2045323	26-12-91
		CN-A-	1057861	15-01-92
		EP-A-	0464287	08-01-92
		JP-A-	6303980	01-11-94
		KR-B-	9512971	24-10-95
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		AU-B-	6860891	02-01-92
		AU-B-	652941	15-09-94
		AU-B-	7925691	02-01-92
		CA-A-	2045323	26-12-91
		CA-A-	2045326	26-12-91
		CN-A-	1057861	15-01-92
		CN-A-	1059758	25-03-92
		EP-A-	0463848	02-01-92
		JP-A-	6303980	01-11-94
		JP-A-	7258290	09-10-95
		KR-B-	9512971	24-10-95
WO-A-9405809	17-03-94	US-A-	5360714	01-11-94
		US-A-	5334525	02-08-94
		AU-B-	5095793	29-03-94
		CA-A-	2143194	17-03-94
		EP-A-	0673437	27-09-95
		JP-T-	8503846	30-04-96
WO-A-9522985	31-08-95	AU-B-	1822395	11-09-95